TRANSGENIC PLANTS AS AN ALTERNATIVE SOURCE OF LIGNOCELLULOSIC-DEGRADING ENZYMES

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FIELD OF THE INVENTION

The present invention is directed to the production of cellulose-degrading enzymes in genetically recombinant plants and the recombinant plants themselves.

BIBLIOGRAPHY

Complete bibliographic citations for the non-patent references discussed hereinbelow are included in the Bibliography section, immediately preceding the claims. All of the references cited below are incorporated herein by reference.

DESCRIPTION OF THE PRIOR ART

Lignocellulosic plant matter, such as agricultural and forestry waste, as well as energy crops produced specifically for biomass, offer tremendous potential for the renewable production of fuel and as chemical feedstocks. However, production cost for desired products such as alcohols from lignocellulosic material is significantly higher than

the production cost of equivalent alternatives. However, the prospect, either real or perceived, of limited fossil fuel reserves, along with the geo-political issues which swirl about petroleum-producing countries and regions, renders the production of basic chemical feedstocks and fuels from local, renewable sources an attractive alternative to fossil fuels.

For instance, alcohols have the potential to be excellent alternative transportation fuels if their production costs can be lowered. Brazil has sponsored several programs to replace car engines which run on gasoline alone to engines which run on ethanol or a gasoline-ethanol mix.

Unfortunately, the production of ethanol and other feedstock chemicals from lignocellulosic material is far more complex than an analogous production utilizing a starch-based starting material. Compared to lignocellulosic materials, starch is a simple polymer which is readily hydrolyzed to glucose. Yeasts can then be used to convert the glucose to ethanol.

In contrast, lignocellulosic biomass is a much more complex substrate in which crystalline cellulose is embedded within a matrix of hemicellulose and lignin. The intricate structure and relative inaccessibility of these substrates requires pre-treatment for the disruption of the lignocellulosic material, as well as hydrolysis of hemicellulose and lignin into xylose and phenolic compounds, respectively. (See, for instance, *Micelli et al.* (1996), *Belkacemi et al.* (1996), and *Grohmann et al.* (1992).)

Several enzymes which degrade lignocellulosic material, commonly referred to as "cellulases," are known. The term "cellulase" shall be used herein to refer to any and all enzymes which catalyze the cleavage of cellulosic or lignocellulosic materials. Explicitly, but not exclusively, included within this definition are those cellulases which fall under the Enzyme Classification heading EC 3.2.1.x. Various genes encoding cellulases have also been isolated and characterized.

For instance, genes which encode endoglucanases from the fungus *Trichoderma reesei* are known and have been successfully incorporated and expressed in yeast. See, for instance, *Pentilla et al.* (1987). Likewise, cellulase E2 (EC 3.2.1.4) and cellulase

E3 (EC 3.2.1.91) from the thermo-tolerant bacterium *Thermomonospora fusca* are known. See *Lao et al.* (1991), *Spezio et al.* (1993) and *Zhang et al.* (1995).

From a functional viewpoint, cellulases are catagorized into two large sub-groups based upon whether they catalyze cleavage from the cellulose chain ends (exocellulases) or if they catalyze cleavage in the middle of the cellulose chain (endocellulases). For instance, cellobiohydrolase I of *T. reesei* (CBH I, EC 3.2.1.91) is an exocellulase, which degrades crystalline cellulose by cleavage from the chain ends. By way of further illustration, CBH I is a 68 kDa protein with a two-domain architecture which is shared by many cellulases. In this chemical architecture, a large catalytic domain is joined to a cellulose-binding domain (CBD) through a flexible linker region. See *Divne et al.* (1994). Similarly, cellulase E3 of *T. fusca* is also an exocellulase.

Different types of cellulases exhibit synergistic activity on complex substrates. This synergism, especially between exocellulases, is believed to be due to differences in their patterns of absorption to and hydrolysis of complex cellulose substrates. See *Henrissat et al.* (1995).

Illustratively, cellulase E2 of *T. fusca* is a 40 kDa endocellulase which cleaves the cellulose chain internally. Such cleavage generates more chain ends for attack by exocellulases. Consequently when CBH I, E2, and E3 cellulases are combined, their activity together is approximately 5-fold greater than their additive individual activities. (See, for instance, *Irwin et al.* (1993) and WO 94/26880.) It is important to note that proteolytic fragments of cellulases can substitute for the intact enzymes in synergistic mixtures. For example, when combined with *T. fusca* E3 and CBH I, the catalytic domain of *T. fusca* E2 ("E2cd") is as active as the intact enzyme in the digestion of filter paper substrate, *Irwin et al.* (1993).

A wide range of compositions containing cellulases are described in the patent literature. For instance, *Evans et al.*, U.S. Patent No. 5,432,074, describe the use of a formulation consisting essentially of a combination of xylanase and xylosidase, but being essentially free of glucanase and cellobiohydrolase. The formulation also contains a lactic acid-producing bacteria. The formulation is used to treat silage to increase its

nutritive value. In operation, the action of the xylanase and xylosidase enzymes degrades non-cellulosic polysaccharides found in the silage material thereby producing sugars for fermentation.

Heterodimers of different types of cellulose-degrading enzymes are described in WO 94/29460. Here, a β-glucosidase molecule and a cellobiohydrolase molecule (*i.e.*, an exocellulase) are chemically bonded to one another by a crosslinking reagent to yield a single molecule which retains the enzymatic activities of the two separate molecules.

Expression constructs which contain cellulase genes for the transformation of yeast have been constructed. For example, *Knowles et al.*, U.S. Patent No. 5,529,919, describe the transformation of *S. cerevisiae* to contain and express a thermostable β-endoglucanase (EG I) of *T. reesei*.

Likewise, attempts have been made to produce transgenic plants which express cellulose-degrading enzymes. *Aspegren et al.* (1995) describe transgenic suspension-cultured barley cells which express EG I of *T. reesei*. The cells were transformed by particle bombardment and transformed cells selected by a co-transformed antibiotic resistance marker. However, no attempt was made to regenerate complete plants from the cultured cells. Of particular note, this reference states that the production of β-glucanases in plant cells may be hampered by the fact that these enzymes catalyze the hydrolysis of essential cell wall components. Attempts by these authors to stably transform tobacco cells with the same construct used to successfully transform the suspended barley cells failed. Here, the authors observed that after transient expression in tobacco protoplasts, cell wall synthesis never resumed.

SUMMARY OF THE INVENTION

The present invention is drawn to genetically recombinant plants which contain one or more exogenous gene sequences which encode one or more cellulose-degrading gene products. The gene product or products are expressed in recoverable quantities in the recombinant plants and can be isolated from the plants, if desired. In the preferred

embodiment, the genetically recombinant plant expresses the gene product constituitively.

However, the invention also encompasses recombinant plants which express the gene product stage-specifically or tissue-specifically. For example, the gene product or products can be expressed in a plant tissue such as the seeds, fruit, leaves, or tubers of the transformed plant host.

The invention is further drawn to recombinant plants as noted above, wherein the plant contains two exogenous genes whose respective gene products are expressed independently of one another. This allows for different types of cellulases to be expressed in different locations within the same recombinant plant. For example, the plant host can be transformed to express two or more heterologous cellulases in different sub-cellular compartments such as the plastid, cytosol, endoplasmic reticulum, mitochondrion, inclusion body, or vacuole. In addition, chloroplast targeting can also be accomplished through the use of direct chloroplast transformation, an approach that circumvents many of the problems associated with expression of heterologous genes in the nuclear genome. *Carrer et al.* (1993), *McBride et al.* (1994).

The invention is further drawn to a method for producing cellulose-degrading enzymes. The method comprises transforming a plant host with one or more exogenous genes which encode one or more cellulose-degrading gene products such that the gene product or products are expressed in recoverable quantities. The plant matter containing the expressed protein can be used directly as a feedstock for biomass conversion, or, if desired, the exogenous enzymes so produced can be isolated and purified.

The cellulases produced by the transgenic plants of the present invention can be utilized in the same manner as conventionally-derived cellulases. For instance, cellulases produced by the transgenic plants of the present invention can be isolated and used in fermentation processes such as brewing and wine-making. Here, the cellulases function to hydrolyze cellulose and β-glucans during fermentation. Or, as described in Example 4, below, whole plants transformed to express cellulases can be used directly or added to ensiled plant matter to increase the extent of fermentation of the ensiled matter. Plants transformed to express functional cellulases may also be fed directly to livestock, where the cellulase activity aids in the digestion of lignocellulosic substrates.

Cellulases produced in the transgenic plants of the present invention can also be utilized in the production of ethanol and other feedstock chemicals from lignocellulosic substrates.

Cellulases produced by transgenic plants of the present invention can also be used in the textile, pulping, and paper-making industries. For instance, cellulases are conventionally used to treat denim fabrics to give them a "stone-washed" appearance. Cellulases are also used to modify paper pulps by digesting the cellulose fibers contained within the pulp. The cellulases produced by the transgenic plants described herein can be used in this fashion.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E2 of *T. fusca*. Promoters and structural genes are depicted as arrows which indicate the direction of transcription. Terminators are depicted as boxes. NPT II = neomycin phosphotransferase; Met-E2m = *T. fusca* E2 (mature form with N-terminal methionine added); MAS-ter = mannopine synthetase terminator; TML-ter = tumor morphology left terminator; MAC-pro = hybrid "MAC" promoter.

Fig. 2 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E3 of *T. fusca*. Promoters and structural genes are depicted as arrows which indicate the direction of transcription. Terminators are depicted as boxes. NPT II = neomycin phosphotransferase; Met-E3m = *T. fusca* E3 (mature form with N-terminal methionine added); MAS-ter = mannopine synthetase terminator; TML-ter = tumor morphology left terminator; MAC-pro = hybrid "MAC" promoter.

Fig. 3 is a western blot analysis evidencing the expression of *T. fusca* E2 cellulase in tobacco transformed to contain the expression construct depicted in Fig. 1.

Fig. 4 is a western blot analysis evidencing the expression of *T. fusca* E3 cellulase

in tobacco transformed to contain the expression construct depicted in Fig. 2.

Fig. 5 is a zymogram gel assay evidencing the expression of active *T. fusca* E2 cellulase in alfalfa transformed to contain the expression construct depicted in Fig. 1.

Fig. 6A is a schematic diagram of a binary vector T-DNA for an expression construct to transform plants to contain cellulase E1 of *A. cellulolyticus*.

Fig. 6B is a schematic diagram of a binary vector T-DNA for an expression construct to transform plants to contain the catalytic domain (E1cd) of cellulase E1 of *A. cellulolyticus*.

Fig. 7 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase CBH I of *T. Reesei*.

Fig. 8A is a plot of data from an activity assay evidencing the expression of *A. cellulolyticus* E1 cellulase in tobacco transformed with Agrobacterium strain PZA8.

Fig. 8B is a plot of data from an activity assay evidencing the expression of *A. cellulolyticus* E1 cellulase in tobacco transformed with Agrobacterium strain PZA9.

Fig. 9 is a western blot analysis evidencing the expression of CBH I cellulase of *T. Reesei* in tobacco transformed to contain the expression construct depicted in Fig. 7.

Fig. 10 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain the CenA endoglucanase of *Cellulomonas fimi*.

Fig. 11 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain endoglucanase D of *Clostridium thermocellum*.

Fig. 12 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain exoglucanase S of *Clostridium cellulovorans*.

Fig. 13 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain exocellulase E6 of *Thermobifida fusca*.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to genetically recombinant plants which express one or more exogenous cellulose-degrading (cellulase) enzymes. The invention is further drawn

to a method of producing cellulases in plants. The invention allows the production of cellulases using the means and methods of large-scale agriculture rather than the conventional route of large-scale fermentation of the bacteria or fungi which are native producers of the cellulases.

The recombinant plants are produced by incorporating into a plant host genome one or more expression constructs comprising a DNA sequence which encodes a protein having cellulose-degrading activity. Introduction of the exogenous gene or genes into the plant is accomplished by any means known to the art. The expression constructs described hereinbelow enable the stable transformation of plants with one or more genes which encode cellulose-degrading enzymes. The constructs include a DNA coding sequence which encodes a cellulase (as that term is described herein) which is operatively linked to regulatory sequences which direct constituitive, stage-specific, or tissue-specific expression of the cellulase DNA.

Cellulose-Degrading Enzymes (Cellulases) and Genes:

As noted above, the term "cellulase" shall be used herein to refer to any and all enzymes which catalyze the cleavage of cellulosic or lignocellulosic materials. As used herein, "cellulase" is synonymous with "cellulose-degrading enzymes." Explicitly, but not exclusively, included within the term cellulases are those enzymes which fall under the Enzyme Classification heading EC 3.2.1.x. A non-exhaustive list of these enzymes, the genes for all of which can be used in the present invention, includes the following:

Table 1: Polysaccharide-Degrading Enzymes

EC 3.2.1.1 (Alpha-amylase)
EC 3.2.1.2 (Beta-amylase)
EC 3.2.1.3 (Glucan 1,4-alpha-glucosidase)
EC 3.2.1.4 (Cellulase, also known as beta-1,4-endoglucanase, *e.g.*, cellulase E2)
EC 3.2.1.6 (Endo-1,3(4)-beta-glucanase)
EC 3.2.1.7 (Inulinase)
EC 3.2.1.8 (Endo-1,4-beta-xylanase)
EC 3.2.1.10 (Oligo-1,6-glucosidase)
EC 3.2.1.11 (Dextranase)
EC 3.2.1.14 (Chitinase)

EC 3.2.1.15 (Polygalacturonase)
EC 3.2.1.17 (Lysozyme)
EC 3.2.1.18 (Exo-alpha-sialidase)
EC 3.2.1.20 (Alpha-glucosidase)
EC 3.2.1.21 (Beta-glucosidase)
EC 3.2.1.22 (Alpha-galactosidase)
EC 3.2.1.23 (Beta-galactosidase)
EC 3.2.1.24 (Alpha-mannosidase)
EC 3.2.1.25 (Beta-mannosidase)
EC 3.2.1.26 (Beta-fructofuranosidase)
EC 3.2.1.28 (Alpha,alpha-trehalase)
EC 3.2.1.31 (Beta-glucuronidase)
EC 3.2.1.32 (Xylan endo-1,3-beta-xylosidase)
EC 3.2.1.33 (Amylo-1,6-glucosidase)
EC 3.2.1.35 (Hyaluronoglucosminidase)
EC 3.2.1.36 (Hyaluronoglucuronidase)
EC 3.2.1.37 (Xylan 1,4-beta-xylosidase)
EC 3.2.1.38 (Beta-D-fucosidase)
EC 3.2.1.39 (Glucan endo-1,3-beta-D-glucosidase)
EC 3.2.1.40 (Alpha-l-rhamnosidase)
EC 3.2.1.41 (Alpha-dextrin endo-1,6-alpha-glucosidase)
EC 3.2.1.42 (GDP-glucosidase)
EC 3.2.1.43 (Beta-L-rhamnosidase)
EC 3.2.1.44 (Fucoidanase)
EC 3.2.1.45 (Glucosylceramidase)
EC 3.2.1.46 (Galactosylceramidase)
EC 3.2.1.47 (Galactosylgalactosylglucosylceramidase)
EC 3.2.1.48 (Sucrose alpha-glucosidase)
EC 3.2.1.49 (Alpha-N-acetylgalactosaminidase)
EC 3.2.1.50 (Alpha-N-acetylglucosaminidase)
EC 3.2.1.51 (Alpha-L-fucosidase)
EC 3.2.1.52 (Beta-N-acetylhexosaminidase)
EC 3.2.1.53 (Beta-N-acetylgalactosaminidase)
EC 3.2.1.54 (Cyclomaltodextrinase)
EC 3.2.1.55 (Alpha-N-arabinofuranosidase)
EC 3.2.1.56 (Glucuronosyl-disulfoglucosamine glucuronidase)
EC 3.2.1.57 (Isopullulanase)
EC 3.2.1.58 (Glucan 1,3-beta-glucosidase)
EC 3.2.1.59 (Glucan endo-1,3-alpha-glucosidase)
EC 3.2.1.60 (Glucan 1,4-alpha-maltotetrahydrolase)
EC 3.2.1.61 (Mycodextranase)
EC 3.2.1.62 (Glycosylceramidase)
EC 3.2.1.63 (1,2-Alpha-L-fucosidase)
EC 3.2.1.64 (2,6-Beta-fructan 6-levanbiohydrolase)

EC 3.2.1.65 (Levanase)
EC 3.2.1.66 (Quercitrinase)
EC 3.2.1.67 (Galacturan 1,4-alpha-galacturonidase)
EC 3.2.1.68 (Isoamylase)
EC 3.2.1.70 (Glucan 1,6-alpha-glucosidase)
EC 3.2.1.71 (Glucan endo-1,2-beta-glucosidase)
EC 3.2.1.72 (Xylan 1,3-beta-xylosidase)
EC 3.2.1.73 (Licheninase)
EC 3.2.1.74 (Glucan 1,4-beta-glucosidase)
EC 3.2.1.75 (Glucan endo-1,6-beta-glucosidase)
EC 3.2.1.76 (L-iduronidase)
EC 3.2.1.77 (Mannan 1,2-(1,3)-alpha-mannosidase)
EC 3.2.1.78 (Mannan endo-1,4-beta-mannosidase)
EC 3.2.1.80 (Fructan beta-fructosidase)
EC 3.2.1.81 (Agarase)
EC 3.2.1.82 (Exo-poly-alpha-galacturonosidase)
EC 3.2.1.83 (Kappa-carrageenase)
EC 3.2.1.84 (Glucan 1,3-alpha-glucosidase)
EC 3.2.1.85 (6-Phospho-beta-galactosidase)
EC 3.2.1.86 (6-Phospho-beta-glucosidase)
EC 3.2.1.87 (Capsular-polysaccharide endo-1,3-alpha-galactosidase)
EC 3.2.1.88 (Beta-L-arabinosidase)
EC 3.2.1.89 (Arabinogalactan endo-1,4-beta-galactosidase)
EC 3.2.1.90 (Arabinogalactan endo-1,3-beta-galactosidase)
EC 3.2.1.91 (Cellulose 1,4-beta-cellobiosidase, also known as beta-1,4-exocellulases; cellobiohydrolases; and exoglucanases; *e.g.*, cellulase E3, CBH I)
EC 3.2.1.92 (Peptidoglycan beta-N-acetylmuramidase)
EC 3.2.1.93 (Alpha,alpha-phosphotrehalase)
EC 3.2.1.94 (Glucan 1,6-alpha-isomaltosidase)
EC 3.2.1.95 (Dextran 1,6-alpha-isomaltotriosidase)
EC 3.2.1.96 (Mannosyl-glycoprotein endo-beta-N-acetylglucosamidase)
EC 3.2.1.97 (Glycopeptide alpha-N-acetylgalactosaminidase)
EC 3.2.1.98 (Glucan 1,4-alpha-maltohexaosidase)
EC 3.2.1.99 (Arabinan endo-1,5-alpha-L-arabinosidase)
EC 3.2.1.100 (Mannan 1,4-beta-mannobiosidase)
EC 3.2.1.101 (Mannan endo-1,6-beta-mannosidase)
EC 3.2.1.102 (Blood-group-substance endo-1,4-beta-galactosidase)
EC 3.2.1.103 (Keratan-sulfate endo-1,4-beta-galactosidase)
EC 3.2.1.104 (Steryl-beta-glucosidase)
EC 3.2.1.105 (Strictosidin beta-glucosidase)
EC 3.2.1.106 (Mannosyl-oligosaccharide glucosidase)
EC 3.2.1.107 (Protein-glucosylgalactosylhydroxylysine glucosidase)
EC 3.2.1.108 (Lactase)
EC 3.2.1.109 (Endogalactosaminidase)

EC 3.2.1.111 (Mucinaminylserine mucinaminidase)
EC 3.2.1.111 (1,3-Alpha-L-fucosidase)
EC 3.2.1.112 (Deoxglucosidase)
EC 3.2.1.113 (Mannosyl-oligosaccharide 1,2-alpha-mannosidase)
EC 3.2.1.114 (Mannosyl-oligosaccharide 1,3-1,6-alpha-mannosidase)
EC 3.2.1.115 (Branched-dextran exo-1,2-alpha-glucosidase)
EC 3.2.1.116 (Glucan 1,4-alpha-maltotriohydrolase)
EC 3.2.1.117 (Amygdalin beta-glucosidase)
EC 3.2.1.118 (Prunasin beta-glucosidase)
EC 3.2.1.119 (Vicianin beta-glucosidase)
EC 3.2.1.120 (Oligoxyloglucan beta-glycosidase)
EC 3.2.1.121 (Polymannuronate hydrolase)
EC 3.2.1.122 (Maltose-6'-phosphate glucosidase)
EC 3.2.1.123 (Endoglycosylceramidase)
EC 3.2.1.124 (3-Deoxy-2-octulosonidase)
EC 3.2.1.125 (Raucaffricine beta-glucosidase)
EC 3.2.1.126 (Coniferin beta-glucosidase)
EC 3.2.1.122 (1,6-Alpha-L-fucosidase)
EC 3.2.1.128 (Glycyrrhizinate beta-glucuroniidase)
EC 3.2.1.129 (Endo-alpha-sialidase)
EC 3.2.1.130 (Glycoprotein endo-alpha-1,2-mannosidase)
EC 3.2.1.131 (Xylan alpha-1,2-glucuronosidase)
EC 3.2.1.132 (Chitosanase)
EC 3.2.1.133 (Glucan 1,4-alpha-maltohydrolase)
EC 3.2.1.134 (Difructose-anhydride synthase)
EC 3.2.1.135 (Neopullulanase)
EC 3.2.1.136 (Glucuronoarabinoxylan endo-1,4-beta-xylanase)
EC 3.2.1.137 (Mannan exo-1,2-1,6-alpha-mannosidase)
EC 3.2.1.138 (Anhydrosialidase)

DNA sequences encoding enzymes having any of the above-described functionalities can be obtained from several microbial sources, including bacterial and fungal sources. Cloning the gene or cDNA sequence of the desired enzyme can be achieved by several well-known methods. A preferred method is to purify the cellulase of interest (or purchase a sample if commercially available) and determine its N-terminal amino acid sequence, as well as several internal amino acid sequences, using known methods. Oligonucleotide probes corresponding to the amino acid sequence are then constructed (again using known methods) and used to screen a genomic or cDNA library of the organism from which the cellulase was isolated. Positive hybrids are identified,

characterized using known methods (restriction enzyme analysis, etc.), and cloned by known means to yield DNA fragments containing the coding sequence for the desired cellulase activity. (See, for instance, *Current Protocols in Molecular Biology*, Chapters 5 and 6.)

If a partial nucleotide sequence of the cellulase of choice is already known, this information can be used to construct suitable primers to directly clone the corresponding cDNA using the polymerase chain reaction (PCR). (See *Current Protocols in Molecular Biology*, Chapter 15.)

Particularly preferred for use in the present invention are those enzymes falling within the classifications EC 3.2.1.4; EC 3.2.1.6; EC 3.2.1.21; and EC 3.2.1.91. The functionality of these particular enzymes is summarized as follows:

EC 3.2.1.4 enzymes (β-1,4-endoglucanases) hydrolyze internal 1,4 glycosidic bonds of the polysaccharide chain, thereby yielding new chain ends at the surface of cellulose crystals.

EC 3.2.1.6 enzymes (β-1,3-endoglucanases) hydrolyze internal 1,3 glycosidic bonds of the polysaccharide chain, which also results in the formation of new chain ends at the surface of cellulose crystals.

EC 3.2.1.21 enzymes (β-glucosidases) hydrolyze cellobiose into glucose, a readily fermentable substrate.

EC 3.2.1.91 enzymes (β-1,4-exocellulases) cleave cellobiosyl residues (cellobiose is a glucose dimer) from the chain ends of cellulose.

Particularly preferred enzymes (and hence particularly preferred genes) for use in the present invention are cellulase E2 and cellulase E3 of *T. fusca* and CBH I of *T. reesei*.

Expression Constructs:

Once the protein coding sequence (*i.e.*, the cellulase gene) has been identified and isolated, it must be inserted into an appropriate expression construct containing regulatory elements to direct the expression of the gene and to direct secretion of the gene product

or targeting of the gene product to a particular sub-cellular location or organelle. Manipulation of oligonucleotide sequences using restriction endonucleases to cleave DNA molecules into fragments and DNA ligase enzymes to unite compatible fragments into a single DNA molecule with subsequent incorporation into a suitable plasmid, cosmid, or other transformation vector are well-known to the art.

A transcription regulatory sequence must be included in the expression construct in order to direct the transformed plant cells to transcribe the inserted cellulase coding sequence. Transcriptional regulators may be inducible or constituitive. Inducible transcription regulators direct transcription of the downstream coding sequences in a tissue-specific or growth-stage specific manner. Constituitive regulators provide for sustained transcription in all cell tissues. For purposes of the present invention, constructs which provide constituitive expression of the coding sequence are preferred.

It is also preferred that the expression construct contain a transcription initiation sequence from the tumor-inducing plasmid (Ti) of *Agrobacterium*. Several T-DNA transcription initiation sequences are well known and include, without limitation, the octopine synthase, nopaline synthase, and mannopine synthase initiators.

Downstream of the initiation sequence and fused to the coding sequence, the expression construct may be manipulated to contain a leader signal sequence which directs the resulting polypeptide to a particular organelle or targets the expressed product for secretion (or to signal post-transcriptional or post-translational modification of the gene product).

Likewise, the expression construct should also include a termination sequence to signal transcription termination.

To facilitate selection of successfully transformed plants, the expression construct should also include one or more selectable markers. The neomycin phosphotransferase gene (NPT II), a well-characterized and widely employed antibiotic resistance selection marker is preferred. This marker provides resistance to kanamycin. A large number of other markers are known and can be used with equal success (*e.g.*, other antibiotic resistance markers, dihydrofolate reductase, luciferase, β-glucuronidase, and the like).

For example, Figs. 1 and 2 depict schematic representations of suitable expression constructs for transformation of plants. These constructs are intended for use with *Agrobacterium*-mediated transformation using the binary vector approach. However, these same constructs can be coated onto micro-projectiles for transformation by particle bombardment. With the exception of the coding sequence, these two constructs are essentially identical: Fig. 1 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E2 of *T. fusca*.

Fig. 2 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E3 of *T. fusca*.

In both Fig. 1 and Fig. 2, promoters and structural genes are depicted as arrows which indicate the direction of transcription and terminators are depicted as boxes. See the "Brief Description of the Figures" for a legend to the abbreviations. In the expression constructs depicted in Figs. 1 and 2, the "MAC" hybrid promoter drives the transcription of the recombinant cellulase genes. Both constructs also contain a constituitive NPT II expression cassette to allow for antibiotic resistance selection using kanamycin. The coding sequence of the construct shown in Fig. 1 (Met-E2m) encodes cellulase E2 from *T. fusca*. (See SEQ. ID. NO: 1; ATG start codon at nt's 255-257, TGA stop codon at nt's 1578-80, first codon of mature E2 protein (AAT) at nt's 348-350.) This sequence encodes the mature form of the enzyme with an N-terminal methionine added. In the same fashion, the coding sequence of the construct shown in Fig. 2 (Met-E3m) encodes cellulase E3 from *T. fusca*. (See SEQ. ID. NO: 2; ATG start codon at nt's 575-577, TAA stop codon at nt's 2363-65, first codon of mature E3 protein (GCC) at nt's 689-692.) This sequence also encodes the mature form of the enzyme with an N-terminal methionine added.

Further examples of constructs which drive targetted expression of cellulose-degrading enzymes are provided in the Examples hereinbelow. Specifically included in the Examples are transformations illustrating apoplastic targeting and accumulation of two additional cellulases. The first of these is the endoglucanase E1 of *Acidothermus cellulolyticus* (EC 3.2.1.4, SEQ. ID. NO: 8). Also included is the cellobiohydrolase

CBH I of *T. Reesei* (EC 3.2.1.91, SEQ. ID. NO: 9). In addition, further Examples of plant expression constructs containing cellulase genes encoding both endoglucanases and cellobiohydrolases (exoglucanase, exocellulase) are provided.

Transformation of Plants:

Transformation of the plants can be accomplished by any means known to the art, including *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and virus-mediated transformation. The method of transformation is not critical to the functionality of the present invention insofar as the method chosen successfully incorporates the oligonucleotide construct containing the cellulase-encoding region and any accompanying regulatory sequences into the plant host. The nature of the plant host to be transformed has some bearing on the preferred transformation protocol. For dicots, *Agrobacterium*-mediated transformation utilizing protoplasts or leaf disks is most preferred. Although the Examples disclose the use of tobacco and alfalfa as bioreactors for cellulase production, any crop plant, including monocots, can be utilized. Transformation of monocots is typically achieved by particle bombardment of embryogenic cell lines or cultured embryos. See, for instance, *Vasil et al.* (1993) and *Castillo et al.* (1994). Recent developments in "super-binary" vectors, however, also allow for the use of *Agrobacterium*-mediated gene transfer in most of the major cereal crops. See, for instance, *Ishida et al.* (1996). In this case, the explant source is typically immature embryos.

Agrobacterium-mediated transformation of the plant host using explants is preferred for its relative ease, efficiency, and speed as compared to other methods of plant transformation. For example, disks are punched from the leaves of the plant host and cultured in a suitable medium where they are then exposed to *Agrobacterium* containing the expression construct and (preferably) a disarmed tumor-inducing (Ti) plasmid. *Agrobacterium tumefaciens* LBA 4404 is the preferred strain for transformation. The preferred binary vector is the pCGN1578 binary vector (*McBride and Summerfelt* (1990)).

The binary vector transformation method is well known and needs only be briefly described herein. See *Zambryski et al.* (1989) for a complete review. The Ti plasmid of *Agrobacterium* contains virulence genes (*vir*) which encode trans-acting proteins that enable the transfer of a portion of the plasmid (the T-DNA) to a plant cell. The T-DNA portion of the Ti plasmid is flanked by two border regions (the right and left borders) which act as recognition sites for the excision of the T-DNA from the plasmid prior to its transfer to the plant host. Excision of the T-DNA is mediated by the *vir* genes of the Ti plasmid and involves nicking of the right and left borders of the T-DNA, which frees a single-stranded oligonucleotide fragment. This fragment is then mobilized out of the *Agrobacterium* and into the plant host target.

In the binary vector method, the T-DNA with its right and left border regions is cloned into *E. coli* in known fashion, and the wild-type genes normally found between the two border regions is excised. The expression construct encoding the cellulase of interest is inserted between the right and left border regions. This construct is designated the "binary plasmid." Construction of the binary plasmid is accomplished utilizing the well-characterized recombinant genetic methods applicable to *E. coli*. Successful transformants are selected utilizing a co-transformed marker appropriate for *E. coli*.

The binary plasmid is then mobilized back into *Agrobacterium*. This is accomplished by direct transformation procedures well known to those skilled in the art.

The *Agrobacterium* itself, such as the preferred LBA 4404 strain, is genetically manipulated to contain a Ti plasmid (called the helper plasmid) which lacks the T-DNA and the tumor-inducing regions (i.e., the Ti plasmid is "disarmed") but which still encodes the virulence proteins necessary for DNA transfer. By cooperation between the helper plasmid and the binary plasmid, the length of DNA between the two border regions of the binary plasmid is excised and mobilized into the plant host, where it is incorporated into the plant host genome. The binary method derives its name from the fact that the plasmid containing the expression construct to be transferred is maintained within *Agrobacterium* as a distinct and independently replicating vector from the Ti plasmid itself.

Selection of successful transformants is accomplished using the co-transformed selection marker discussed above. If the marker is NPT II, selection is accomplished by treatment with kanamycin.

For the present invention, the most preferred plants for transformation are alfalfa and tobacco. However, any plant species will function with comparable success. Included among the plant species which can be utilized in the present invention are cauliflowers, artichokes, apples, bananas, cherries, cucumbers, grapes, lemons, melons, nuts, oranges, peaches, pears, plums, strawberries, tomatoes, cabbages, endive, leeks, lettuce, spinach, arrowroot, beets, carrots, cassava, turnips, radishes, yams, sweet potatoes, beans, peas, soya, wheat, barley, corn, rice, rapeseed, millet, sunflower, oats, tubers, kohlrabi, potatoes, and the like.

The plants to be transformed are preferably common green field plants, such as the preferred alfalfa and tobacco, as well as soya, corn, and the like. Equally preferred are plant hosts which are grown specifically for "biomass energy," such as switchgrass, poplar, and the like. In this instance, the enzymes would not be recovered from the plants. The plants are then transformed and regenerated into whole plants which express fully-functional, cellulose-degrading enzymes in economically significant quantities. Alfalfa is one of the most preferred plant species for use in the present invention because alfalfa is a hardy, perennial plant, which grows well with minimal fertilization and irrigation. Alfalfa is also a very prolific plant. In temperate areas such as those found in the midwestern United States, alfalfa will yield three or more harvests per growing season. Methods have also been developed for wet fractionation of the herbage matter to recover value-added products therefrom.

Tobacco is equally preferred for its prolific growth, ease of transformation, and its well-characterized genetics. Both alfalfa and tobacco are widely cultivated throughout the United States and in other parts of the world.

In the most preferred embodiment, alfalfa or tobacco plants are stably transformed to express, constituitively, enzymatically active E2 or E3 cellulases from *T. fusca*. Also preferred are alfalfa or tobacco which express enzymatically active CBH I from *T. reesei* or combinations of E2, E3, and CBH I. The *T. fusca* cellulases are most preferred

because they are native to thermo-tolerant bacteria and are relatively heat stable. This allows isolation of the cellulase from plant material using relatively rigorous heat precipitation without adversely effecting the activity of the cellulase.

Stage-Specific and Tissue-Specific Expression of Cellulases:

Because the enzymes to be expressed by the transformed plant hosts hydrolyze components of the plant cell wall, high levels of expression might have a deleterious effect on the plant host. Therefore, targeting of the expressed enzyme to particular sub-cellular compartments may be preferred. Targeting of the expressed enzyme may also be preferred to avoid expression of the enzyme in sub-cellular compartments where proteolytic activity is high. Targeting of the expressed enzyme may also be preferred if the exogenous cellulase activity interferes with the normal cellular metabolism of certain compartments.

For instance, targeting expression to the apoplast allows the exogenous protein to avoid the more active protein-degrading systems of other cellular compartments, such as in plant leaf vacuoles.

Several signal sequences are known and can be utilized in the present invention. For example, signal sequences for targeting to the secretory pathway are known, *Wandelt et al.* (1992), *Bednarek* (1991), *Mason et al.* (1988), as are sequences for targeting to the chloroplast, *Keegstra et al.* (1993), and the mitochondrion, *de Castro Silva Filho et al.* (1996).

For apoplast targeting, the VSP leader is preferred. The VSP leader comprises the amino acid sequence: NH_3-Met-Lys-Leu-Phe-Val-Phe-Phe-Val-Ala-Ala-Val-Val-Leu-Val-Ala-Trp-Pro-Cys-His-Gly-Ala- (SEQ. ID. NO: 3). See *Mason et al.* (1988).

Additionally, bacterial secretory sequences found in the wild-type cellulase gene may be removed to afford cytoplasmic expression of the enzyme in the recombinant plant host.

Targeting can be achieved by fusing combinations of mitochondrial and chloroplast targeting signals to the N-terminus of the desired cellulase, as has been

demonstrated for the reporter genes chloramphenicol acetyl transferase and β-glucuronidase, *de Castro Silva Filho et al.* (1996). In some cases, efficient translocation requires the presence of both signal peptides, with the amino terminal peptide being crucial in specifying import into a particular organelle. In addition, vacuole targeting can be achieved by fusing the sequence encoding the N-terminal 146 amino acids of the vacuolar patatin protein between a secretory leader and structural gene for the cellulase, as has been demonstrated for the yeast invertase gene, *Sonnewald et. al.* (1991).

Regeneration of Mature Transgenic Plants:

Transgenic tobacco and alfalfa were produced by *Agrobacterium*-mediated transformation using explants as source material. This is a routine method easily followed by those skilled in the art. The production methods for transgenic tobacco and alfalfa are given as non-limiting illustrations of the practice of the invention.

The transformation procedure for tobacco is essentially the explant method developed by Horsh et al. (1985). Leaf explants are taken from the second and third fully expanded leaves of three-week old *in vitro* shoot cultures of *Nicotiana tabacum* W38 maintained on MS medium, *Murashige and Skoog* (1962). The leaf pieces are cut into 1 cm squares and pre-cultured on MS medium with 2.0 mg/L 6-benzyl-aminopurine (BAP) and 0.1 mg/L alpha-naphthalene acetic acid (NAA) for 24 hours at 25°C with a 16 hour photo period of 70-90 μE $m^{-2}s^{-1}$. After pre-culture, explants are placed into a suspension of *Agrobacterium* cells. After 30 minutes, leaf explants are blotted on filter paper and placed abaxial-side down on MS medium with 1.0 mg/L BAP and 0.1 mg/L NAA and co-cultivated for four days under the same conditions as given above. Leaf pieces are then rinsed three times in sterile water, blotted on filter paper, and transferred to the media used for co-cultivation but containing 100 mg/L kanamycin and 400 mg/L carbenicillin. Plantlets (typically 2-3) develop 10-14 days later from callus formed along cut leaf edges. If desired, further plantlet formation can be achieved by transfer of explants to fresh medium at two week intervals. Plantlets are excised and rooted on MS media containing 100 mg/L kanamycin and 400 mg/L carbenicillin.

To transform alfalfa, new-growth trifoliates are taken from alfalfa plants (regenerable genotypes, *Bingham et al.* (1975)) maintained in a growth room and sterilized using alcohol and bleach washes (30 seconds in 70% alcohol, 90 seconds in 20% hypochlorite + 0.1% SDS, followed by three rinses in sterile distilled water). Leaf edges are cut on moist filter paper and tissue then placed into liquid SH-II medium. (*Bingham et al., supra.*) When sufficient explants have been taken, the explants are moved to a suspension of *Agrobacterium* cells containing the engineered plasmid. (The *Agrobacterium* suspension is taken from an overnight culture grown in liquid YEP selection medium.) Cell density is adjusted to fall between about 0.6 to about 0.8 at A_{660}. After 30 minutes inoculation, the explants are gently blotted on filter paper and placed on B5H medium, *Brown and Atanassov* (1985), for four days. They are then rinsed twice in sterile water and cultured on B5H for a further four days. At the end of this period, they are rinsed three times and transferred to B5H containing 25 mg/L kanamycin and 250 mg/L carbenicillin. Plates are maintained at 24°C, 16 hour photo period, light intensity 60-80 μE $m^{-2}s^{-1}$. Explant-derived calli (and occasionally embryoids) which form within 3 weeks on this medium are moved to B5H with antibiotics but without hormones to allow for further embryoid production and development of existing embryoids. After three to four weeks, embryos are transferred to MS medium including the two antibiotics to allow for development into plantlets. Callus forms on untreated explants in the presence of 25 mg/L kanamycin but embryos are never produced. Each explant piece can give rise to multiple (up to 40) embryos. Plantlets are rooted on MS medium lacking antibiotics.

Monitoring Cellulase Expression:

Cellulase expression can be monitored using a number of different methods, the two most common being western blot analysis (which detects cellulase protein using antibodies specific for the cellulase of interest) and zymographic analysis or enzyme assay (both of which measure the ability of the expressed cellulase to degrade a cellulosic substrate).

Briefly, in the western blot technique, whole plant samples (or root tips, leaves, etc.) are ground in an extraction buffer (preferably 50 mM sodium acetate (pH 5.5) and 10 mM dithiothreitol) and an aliquot of the extract loaded onto an electrophoresis gel (*e.g.*, polyacrylamide containing SDS). Preferably, identical extractions are performed on non-transformed plants and aliquots of these extractions are then loaded onto parallel lanes of the gel to act as negative controls. Serial dilutions of purified cellulase standards can be also electrophoresed to act as positive controls. The gel is then subjected to electrophoresis in standard and well known fashion.

After electrophoresis is complete, the separated proteins are electro-transferred to a nitrocellulose, PVDF, or nylon membrane, in well known fashion. The membrane containing the immobilized proteins is then immersed in a non-specific blocking buffer or detergent (*e.g.*, "TWEEN 20"), and then placed in a solution containing an antibody (the primary antibody) which is specifically reactive with the particular cellulase under investigation. The membrane is then washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (*e.g.*, goat anti-rabbit IgG). The membrane is then exposed to a chromogenic or luminescent substrate to visualize cellulase hybridization on the membrane.

Zymograms in which the cellulase of interest is resolved in a gel system and then assayed for activity within the gel provide a relatively simple way to assess the activity of cellulases in crude cell lysates. See *Coughlan* (1988). In this approach, plant tissue is ground in the presence of an appropriate grinding buffer (100 mM Tris-HCl pH 9.0, 5 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM ethylenediamine-tetraacetic acid, for example). After grinding of the tissue, an equal volume of a 50% (v/v) slurry of washed polyvinylpolypyrrolidone (suspended in grinding buffer) is added and mixed thoroughly. After centrifugation of the mixture, a sample of the cleared extract is subjected to electrophoresis through a non-denaturing (8%, w/v) polyacrylamide gel. The resulting gel is used to prepare a sandwich with a thin film (<2 mm) of agarose (0.7 % agarose, 0.5 % Sigma medium viscosity carboxymethycellulose) bonded to "GELBOND" film (FMC Corporation). After incubation for 1.5 hours at 50° C, the agarose film is stained with "CONGO RED" dye

for 30 minutes followed by a 1M NaCl wash. After several minutes, it is possible to visualize cellulase activity as a clear zone within a background of red staining.

Cellulase activity is most commonly assayed in aqueous solution, using a cellulosic substrate and monitoring the reaction for either the release of a chromophore/fluorophore or release of cellobiose ("reducing sugar"). For example, *T. fusca* E2 activity can be measured by incubating a sample of the enzyme in a 0.4 ml reaction containing 1% (w/v) low viscosity carboxymethylcellulose (Sigma C-5678) and 50 mM NaOAc pH 5.5 at 55°C for 2-20 hours. 1.0 ml of DNS solution, see *Irwin et al.* (1993), is then added and the mixture is boiled for 15 minutes. Measurement of absorbance values at 600 nm for each reaction can then be correlated to values determined for a known series of glucose standards to determine the extent of carboxymethylcellulose hydrolysis. For plant extracts, background values are determined by preparing parallel reaction samples which contain no substrate and subtracting this value from that obtained in the presence of 1% carboxymethylcellulose.

For a more complete discussion of cellulase assays, see *Adney et al.* (1994), *Baker et al.* (1992), *Tucker et al.* (1989) and *Irwin et al.* (1993).

Isolation of Cellulase Activity from Plants:

It is most preferred that, where applicable, the enzyme not be purified from the plant material, but rather that the plant material containing the cellulase activity be used directly. This is demonstrated in the Examples, below, where transgenic alfalfa which expressed cellulase activity is added directly to silage materials to further the extent of fermentation.

If isolation of the cellulase activity is desired, this can be accomplished by any means known to the art. For example, the preferred *T. fusca* E2, E3, and CBH I enzymes are taken from thermo-tolerant bacteria. The activity of these enzymes remains unchanged by treatments up to about 55-60°C. Therefore, these enzymes can be isolated by gently heating the plant material in aqueous buffered solution (100 mM Tris/HCl pH 9.0, for example) to precipitate the bulk of plant proteins. The soluble cellulase enzymes

are then recovered and further purified by any means known to the art, including HPLC, affinity chromatography, and the like. To facilitate downstream processing of the enzyme, a purification tag may optionally be incorporated into the expressed cellulase.

Since the above-mentioned enzymes are well-characterized, the preferred purification scheme is based on established protocols already in existence. For example, *T. fusca* E2 from a heat-treated plant extract is further purified by adsorption to a phenyl "SEPHAROSE" column in the presence if 0.8 M ammonium sulfate. Successive column washes using ammonium sulfate concentrations of 0.6 M and 0.3 M in a buffer containing 5 mM KPi, pH 6.0 and 5 mM NaCl are followed by a final wash with 0.1 M KPi, pH 6.0. Elution of E2 is accomplished using 5 mM KPi, pH 6.0. Peak fractions are loaded on a hydroxylapatite column (equilibrated with 1 mM KPi, pH 6.0) and the flow-through fractions collected and pooled. The pooled fractions are loaded on a "Q-SEPHAROSE" column (pre-equilibrated with 10 mM BisTris, pH 6.0) and eluted with a continuous buffer gradient from 5 mM BisTris, pH 6.0 to 150 mM NaCl, 5 mM BisTris, pH 6.0. Peak fractions from the "Q-SEPHAROSE" column are then pooled, concentrated and stored frozen in convenient aliquots. Similarly detailed protocols exist for both *T. fusca* E3 and *T. reesei* CBH I, see *Irwin et al.* (1993).

EXAMPLES

The following Examples are included solely to aid in a more complete understanding of the manufacture and use of the transgenic plants disclosed and claimed herein. The Examples do not limit the scope of the invention in any fashion.

Example 1: Production of Transgenic Alfalfa and Tobacco Which Express Cellulase "E2" of *T. fusca*

Transgenic alfalfa and tobacco plants were produced using the same protocol. Binary vectors carrying recombinant cellulase expression cassettes were transformed into *Agrobacterium tumefaciens* strain LBA 4404, facilitating *Agrobacterium*-mediated

transformation of plant tissue. The construct used is shown in Fig. 1. The gene encoding the E2 cellulase of *Thermomonospora fusca* was obtained as described by *Ghangas & Wilson* (1988). The E2 gene was modified by PCR using the XbaE2 primer, 5'-GC***TCTAGA***ATGAATGATTCTCCGTTC-3' (SEQ. ID. NO: 4) and the "-20 sequencing primer," 5'-TGACCGGCAGCAAAATG-3' (SEQ. ID. NO: 5), (product #1211, New England Biolabs, Inc., Beverly, Massachusetts), resulting in a recombinant gene in which an *Xba I* site (***bold italics***) was incorporated immediately 5' to an introduced start codon (underlined). This start codon precedes the first codon encoding the mature form of the E2 protein (AAT, nt's 348-350 in SEQ. ID. NO: 1). The net effect of these changes is the removal of the bacterial secretion signal peptide (resulting in cytosolic accumulation), the addition of a novel cloning site to facilitate expression cassette construction and the addition of a methionine residue to the N-terminus of the protein compared to the processed mature form of E2 obtained from *T. fusca*).

The cloned E2 gene required no modification at the 3' end as a convenient *Eco RI* restriction site occurs approximately 45 nucleotides 3' to the stop codon.

The preferred expression cassette includes the hybrid "MAC" promoter and the mannopine synthetase terminator. The MAC promoter contains distal elements, including the transcriptional enhancer, of the CaMV 35S promoter (-940 to -90, relative to the mRNA start site), as well as proximal promoter elements derived from the *Agrobacterium* mannopine synthetase promoter (-301 to +65 relative to the mRNA start site). MAC has been reported to result in higher levels of expression than either of the natural promoters (*Comai et al.* (1990).) The expression cassette was cloned into the pCGN1578 binary vector and mobilized into *Agrobacterium*.

Initial tobacco transformants were screened by western blot to determine the level of expression. Levels of expression ranged up to 0.1 to 0.2 % of extracted protein. The mature plants were allowed to self and set seed. One of the initial transformants, designated CT30, was tested further to verify the sexual transmission of the transgene. Sl seeds from this plant were germinated and tested for kanamycin resistance. Leaf samples from kan^R seedlings as well as a W38 control were prepared for western blot

analysis as described previously. The results are depicted in Fig. 3. Each lane contained extract corresponding to 5 mg fresh weight of leaf tissue. In addition, 1 ng of purified E2 enzyme was loaded as a control. Levels of expression were similar to that observed in the parental transformant, demonstrating the stable sexual transfer of this trait. Similar genetic stability was also observed in alfalfa plants transformed with this transgene.

The thermal stability and enzymatic activity of recombinant *T. fusca* E2 was demonstrated using transgenic alfalfa. Samples were prepared for zymogram analysis as described above. As shown in Fig. 5, aliquots of alfalfa extract were treated for 5, 10 and 20 minutes (grouped from left to right) at each of the temperatures indicated (°C) before being subjected to native gel electrophoresis. An untreated sample of extract and two purified E2 standards were included as controls. Levels of E2 activity corresponded well with expected activity based on western blot analysis of samples from the same plant. In addition, no significant loss in band intensity (activity) was observed at any of the treatment temperatures, despite the fact that greater than 95% of the soluble protein in the extract is denatured after 20 minutes at 65°C.

Example 2: Production of Transgenic Alfalfa and Tobacco Which Express Cellulase "E3" of *T. fusca*

Here, the expression construct depicted in Fig. 2 was used to transform alfalfa and tobacco using the same methodology as described in Example 1.

The gene encoding the E3 cellulase of *Thermomonospore fusca* was obtained as described by *Zhang et al.* (1995). The 5' end of the E3 gene was modified by PCR using the primer XbaE3, 5'-GC***TCTAGA***ATGGCCGGCTGCTCGGTG-3' (SEQ. ID. NO: 6), resulting in a recombinant gene in which an *Xba I* site (***bold italics***) was incorporated immediately 5' to an introduced start codon (underlined). This start codon precedes the first codon encoding the mature form of the E3 protein (GCC, nt 689-691 in SEQ. ID. NO: 2). The 3' end of the E3 gene was modified using the primer RIE3, 5'-G***GAATTC***TTACAGAGGCGGGTAG-3' (SEQ. ID. NO: 7), thereby placing an *Eco RI*

restriction site (***bold italics***) immediately 3' to the stop codon (<u>underlined</u>) for the E3 gene. Note that this latter primer is homologous to the noncoding strand of the E3 gene. The net effect of these changes is the removal of the bacterial secretion signal peptide (resulting in cytosolic accumulation), the addition of novel cloning sites to facilitate expression cassette construction and the addition of a methionine residue to the N-terminus of the protein (compared to the processed, mature form of E3 obtained from *T. fusca*).

The E3 expression cassette was constructed as described above for the E2 cassette.

Initial tobacco transformants were screened by western blot to determine the level of expression. Levels of expression ranged up to about 0.04% of extracted protein. The mature plants were allowed to self and set seed. One of the initial transformants, designated CT117, was tested further to verify the sexual transmission of the transgene. S1 seeds from this plant were germinated and tested for kanamycin resistance. Leaf samples from kan^R seedlings as well as a W38 control were prepared for western blot analysis as described previously. The results are depicted in Fig. 4. Each lane contained extract corresponding to 5 mg fresh weight of leaf tissue. In addition, 1 ng of purified E3 enzyme was loaded as a control. Levels of expression were comparable to that observed in the parental transformant, demonstrating the stable sexual transfer of this trait.

Example 3: Sexual Transfer of Cellulase Expression in Tobacco and Alfalfa

Original transgenic lines of tobacco and alfalfa shown to express either E2 or E3 cellulase were used in sexual crosses. In both cases, the trait segregated in progeny as predicted by Mendelian genetics. Expression levels were the same as, or greater than those seen in parental lines.

Example 4: Use of Transgenic Alfalfa in Silage

Here, regular non-transformed alfalfa, alfalfa transformed according to Example 1 and alfalfa transformed according to Example 2 were ensiled under identical conditions

for one month and the products of fermentation for each experiment quantified. The results are presented in Table 2.

All of the ensiled plant material was ground separately through a manual meat grinder. The grinder was rinsed with water and wiped with ethanol after grinding each sample. A 1 to 1 to 1 mixture of non-transformed alfalfa, E2-transformed alfalfa, and E3-transformed alfalfa was ground together and used to assemble two control silos (Cont1 and Cont2, 50 g each). The two control silos were inoculated with a 1 mL of a commercial inoculant (0.1098 g "BIOMATE LP/PC" concentrate in 500 mL sterile water).

Two silos each of E2-transformed alfalfa (E2-1, E2-2) and E3-transformed alfalfa (E3-1, E3-2) were constructed in the same fashion as the controls (35 g each, inoculated with 0.6 mL of the above-noted inoculant). Two silos of mixed E2- and E3-transformed alfalfa were constructed by grinding together 17.5 g each of E2- and E3-transformed alfalfa per silo (35 g each, inoculated with 0.6 mL of the above-noted inoculant).

All of the silos were then placed into a 30°C water bath until opening.

Of special note in this Example is the increased amount of fermentation products in the transgenic alfalfa as compared to the non-transformed alfalfa. In particular, note that a mixture of alfalfa herbage expressing both the E2 and E3 cellulases exhibits markedly improved fermentation yield as compared to the non-transformed alfalfa and ensiled alfalfa expressing either E2 or E3 enzymes.

Clearly, as shown by this Example, expression of cellulases in transgenic alfalfa leads to better silage production.

TABLE 2

					Organic Acid Analysis (OAA) via HPLC							
Sample												**Avg.**
ID	**%DM**	**pH**	**SUC**	**LAC**	**FOR**	**ACE**	**PRO**	**2,3But**	**ETOH**	**BUT**	**Total Prod.**	**Total Prod.**
Cont 1	23.33612	5.741	0.144	2.014	0.000	1.764	0.000	0.239	0.704	0.000	4.86	5.47
Cont 2	21.62983	5.121	0.159	3.885	0.000	1.407	0.328	0.000	0.294	0.000	6.07	
E2-1	22.99369	5.277	0.157	3.390	0.000	2.300	0.233	0.000	0.330	0.000	6.41	6.09
E2-2	23.83774	5.166	0.361	2.935	0.000	1.998	0.177	0.000	0.298	0.000	5.77	
E3-1	22.88773	5.128	0.283	3.321	0.000	2.380	0.177	0.000	0.292	0.000	6.45	6.61
E3-2	22.22822	5.151	0.354	3.324	0.000	2.608	0.200	0.000	0.288	0.000	6.77	
E23-1	22.95945	5.743	0.551	2.848	0.000	3.185	0.328	0.000	0.337	0.000	7.25	7.45
E23-2	22.66411	5.888	0.602	2.745	0.000	3.649	0.301	0.000	0.353	0.000	7.65	

The table headings are as follows:

%DM = percent dry matter of silage, pH = acidity, SUC = succinic acid, LAC = lactic acid, FOR = formic acid, ACE = acetic acid, PRO = propionic acid, 2,3 But = 2,3-butanediol, ETOH = ethanol, BUT = butyric acid, Cont 1 and Cont 2 = controls, E2-1 and E2-2 = transformant expressing E2, E3-1 and E3-2 = transformants expressing E3, E23-1 and E23-2 = a 1:1 mixture of herbage from transgenic alfalfa expressing E2 and E3.

Example 5: Production of Transgenic Tobacco Plants Which Express Cellulase "E1" of *A. cellulolyticus*

Transgenic tobacco plants were produced in a manner analogous to that described in Examples 1 and 2. Binary vectors carrying recombinant cellulase expression cassettes were transformed into *Agrobacterium tumefaciens* strain LBA 4404, thereby facilitating *Agrobacterium*-mediated transformation of plant tissue. The constructs for this Example are depicted schematically in Figs. 6A and 6B. The gene encoding the E1 cellulase of *Acidothermus cellulolyticus* was obtained as described previously by *Himmel et al.* (see U.S. Patent No. 5,275,944). The E1 gene was then modified by PCR using the *Nar*E1 primer, 5'-CGGGGCGCCGGCGGCGGCTAT-3'(SEQ. ID. NO: 10) and the *Sac*E1 primer, 5'-CCGAGCTCTTAACTTGCTGC-3' (SEQ. ID. NO: 11) to generate a recombinant E1 gene. The recombinant gene has a *Nar*I site at the 5' end and a *Sac*I site at the 3' end (restriction sites are underlined) to facilitate fusion to the VSP leader coding sequence (SEQ. ID. NO: 3) and nopaline synthetase terminator.

As in the previous Examples, PCR-derived fragments were sequenced to verify that no errors (mutations) had been introduced. The resulting cassette includes the VSP leader sequence operationally linked to the "mature" portion of the E1 coding sequence. This cassette was then cloned into pBI121 (Clontech Labs, Palo Alto, California) as a *Xba*I to *Sac*I fragment, replacing the *uidA* gene and placing the new construct (designated pZ49.1) under the control of the CaMV 35S promoter.

An analogous construct (designated pZ57.1) was generated in which the E1 coding sequence was truncated to yield the E1 catalytic domain (E1cd) using the *Nar*E1 primer (SEQ. ID. NO: 10) and the *Sac*E1cd primer, 5'-TGGAGCTCTA***GAC***AGGATCGAAAAT-3' (SEQ. ID. NO: 12). This construct encodes a polypeptide containing the VSP leader peptide (SEQ. ID. NO: 13) fused to the first 358 amino acids of the E1 protein. The codon specifying valine 358 is bold, italics (note that this oligonucleotide represents the "antisense" strand). Plasmids pZ49.1 and pZ57.1 were transformed into *Agrobacterium tumefaciens* strain LBA4404 to yield strains PZA8 and PZA9, respectively.

Putative transgenic tobacco plants were screened by a combination of Western blotting and E1 activity assay. Leaf samples were removed from plants grown in Magenta boxes (MS medium). Samples were ground in E1 grinding buffer (50 mM NaOAc pH 5.5, 100 mM NaCl, 10% (v/v) glycerol, 1 mM ethylenediamine-tetraacetic acid, 1 mM phenylmethanesulfonyl fluoride, 1 mg/l aprotinin, 1 mg/l leupeptin, 1 mg/l pepstatin), added at a ratio of 2 μl per mg of sample. Samples of extract were centrifuged at >10,000 x G for 5 minutes to remove insoluble material and diluted 100-fold in E1 grinding buffer to which acetylated bovine serum albumin had been added (0.1 mg/ml final concentration). Diluted extract was assayed for activity at 65°C using β-D-cellobiopyranoside (MUCB) as a substrate (0.5 mM MUCB, 50 mM NaOAc pH 5.5, 100 mM NaCl).

Reactions were terminated by the addition of an equal volume of 150 mM glycine/NaOH (pH 10). Fluorescence at 460 nm was quantified using a commercial plate reader ("BIOLUMIN 960," Molecular Dynamics) with excitation set at 355 nm. Enzyme activity in extracts was then compared to the activity of purified E1 holoenzyme and E1 catalytic domain (generously provided by Steve Thomas, National Renewable Energy Laboratory, Golden, Colorado).

In addition, a set of 4-methylumbelliferone standards was also assembled for use as calibration standards. The same extracts were also subjected to analysis by Western blotting. Both PZA8- and PZA9-transformed tobacco plants accumulated an immunoreactive species that co-migrates with purified E1cd. Very little full-length E1 is present in PZA8 transformants, indicating that proteolytic processing of the E1 enzyme is taking place. For this reason, activities are reported as E1cd equivalent, even in those plants that contain an intact E1 coding sequence. For PZA8 transformants, the average E1 expression level was 0.10% of total soluble protein, with the highest expressing plant accumulating E1 at 0.33% of total soluble protein (see Fig. 8A). E1 expression was higher in PZA9 transformants, with an average expression level of 0.21% of total soluble protein and a high value of 0.59% (See Fig. 8B).

Example 6: Production of Transgenic Tobacco Plants Which Express Cellobiohydrolase I (CBH I) of *Trichoderma reesei*

Transgenic plants were generated essentially as described in Example 5. A schematic diagram of the construct used is shown in Fig. 7. The gene encoding CBH I of *Trichoderma reesei* (SEQ. ID. NO: 9) was generously provided by Steve Thomas (National Renewable Energy Laboratory, Golden, Colorado) and is substantially the same as the gene described by *Shoemaker et al.* with its introns removed. Sequence data obtained by the inventors and by NREL scientists indicates that the gene used in this Example differs from the *Shoemaker et al.* sequence at nucleotide 1429. Specifically, the gene used here contains a 4 base-pair sequence (CGCC) inserted in place of G1429, thereby effectively inserting an additional codon and replacing Arg459 with two prolines. A similar substitution exists in a related CBH I enzyme from *Trichoderma viride* (see *Cheng et al.*, 1990). The gene used here also has a silent mutation in the codon specifying Thr41 (ACT changed to ACG).

The CBH I gene was modified by PCR using the cbh2-2 primer, 5'-GCTCTAG***ATG***TATCGGAAGTTGGC-3' (SEQ. ID. NO: 14) and the cbh3-1 primer, 5'-CCCCCGGGTTACAGGCACTGAGAG-3' (SEQ. ID. NO: 15) to generate a recombinant CBH I gene which retains its secretory leader peptide. The recombinant gene has an *Xba*I site at the 5' end and a *Xma*I site at the 3' end (restriction sites are shown in underline, start codon is bold, italics) to facilitate vector construction. The gene was cloned into pBI121 (Clontech) as a *Xba*I to *Xma*I fragment, replacing the *uidA* gene and placing the CBH I gene under the control of the CaMV 35S promoter.

Putative transgenic tobacco plants were screened by Western blotting. Leaf samples were removed from plants grown in Magenta boxes (MS medium) and ground in E1 grinding buffer (see composition in Example 5), added at a ratio of 2 μl per mg of sample. The extract was centrifuged at >10,000 x G for 5 minutes to remove insoluble material and a portion prepared for SDS-PAGE and subsequent blotting and detection. See Fig. 9, which depicts the SDS-PAGE gel. Of 12 plants screened in this way, 3 had detectable expression (about 0.01% of total soluble protein). In addition,

immunoreactive material migrated slightly ahead of the RuBisCo large subunit, consistent with the expected mobility of the 48 kDa catalytic domain (*Divne et al.*, 1994). This suggests that CBH I, like *A. cellulolyticus* E1, is proteolytically cleaved by a plant protease.

Example 7: Production of Transgenic Tobacco Plants Which Express the Endoglucanase Encoded by the *cenA* Gene of *Cellulomonas fimi*

In a manner analogous to the previous Examples, the *cenA* gene of *Cellulomonas fimi* (*Wong et al.*, 1986) can be expressed in tobacco. A schematic diagram of the construct used is shown in Fig. 10. The *cenA* gene (SEQ. ID. NO: 16) is modified by PCR using the *cenA*pst primer, 5'-GGCTGCA***GGC***GGCTGCCGCGTCGAC-3' (SEQ. ID. NO: 17) and the *cenA*sac primer, 5'-CCGAGCTCTCACCACCTGGCGTT-3' (SEQ. ID. NO: 18) to generate a recombinant *cenA* gene. The recombinant gene has a *Pst*I site at the 5' end and a *Sac*I site at the 3' end (restriction sites are underlined, novel glycine codon in bold italics) to facilitate fusion to a VSP leader coding sequence (SEQ. ID. NO: 19) and nopaline synthetase terminator. In addition, the proline at position 2 in the mature endoglucanase enzyme is changed to a glycine, a conservative substitution. The resulting cassette consists of the VSP leader sequence fused to the "mature" portion of the *cenA* gene.

This cassette is then cloned into pBI121 (Clontech) as a *Xba*I to *Sac*I fragment, replacing the *uidA* gene and placing the new construct under the control of the CaMV 35S promoter. As in the previous Examples, *Agrobacterium tumefaciens* strain LBA4404 is transformed with the resulting binary vector and subsequently used to transform plants.

Example 8: Production of Transgenic Tobacco Plants Which Express Endoglucanase D, Encoded by the *celD* Gene of *Clostridium thermocellum*

In a manner analogous to the previous Examples, the *celD* gene of *Clostridium thermocellum* (see *Joliff et al.*, 1986) can be expressed in tobacco. A schematic diagram

of the construct used is shown in Fig. 11. The *celD* gene (SEQ. ID. NO: 20) is modified by PCR using the *celD*pst primer, 5'-AGCTGCAGAAATAACGG-3' (SEQ. ID. NO: 21) and the *celD*sac primer, 5'-CCGAGCTCTTATATTGGTAATTTCTC-3' (SEQ. ID. NO: 22) to generate a recombinant *celD* gene. The recombinant gene has a *Pst*I site at the 5' end and a *Sac*I site at the 3' end (restriction sites are underlined) to facilitate fusion to the VSP leader coding sequence (SEQ. ID. NO: 19) and nopaline synthetase terminator. The resulting cassette includes the VSP leader sequence fused to the "mature" portion of the celD gene. Subsequent manipulations are be carried out as described in the previous Examples.

Example 9: Production of Transgenic Tobacco Plants Which Express Exoglucanase S, Encoded by the *exgS* Gene of *Clostridium cellulovorans*

In a manner analogous to the previous Examples, the *exgS* gene of *Clostridium cellulovorans* (see *Liu and Doi*, 1998) can be expressed in tobacco. A schematic diagram of the construct used is shown in Fig. 12. The *exgS* gene (SEQ. ID. NO: 23) is modified by PCR using the *exgS*nar primer, 5'-CGGGGCGCCGCACCAGTAGTGCCA-3' (SEQ. ID. NO: 24) and the *exgS*sac primer, 5'-CCGAGCTCTTATTTAATCTTAAGC-3' (SEQ. ID. NO: 25) to generate a recombinant *exgS* gene. The recombinant gene has a *Nar*I site at the 5' end and a *Sac*I site at the 3' end (restriction sites are underlined) to facilitate fusion to the VSP leader coding sequence (SEQ. ID. NO: 13) and nopaline synthetase terminator. The resulting cassette consists of the VSP leader sequence fused to the "mature" portion of the *exgS* gene. Subsequent manipulations are carried out as described previously.

Example 10: Production of Transgenic Tobacco Plants Which Express Exocellulase E6, Encoded by the *celF* Gene of *Thermobifida fusca* (formerly *Thermomonospora fusca*)

In a manner analogous to the previous Examples, the *celF* gene of *Thermobifida fusca* (see *Irwin et al.*, 1999) can be expressed in tobacco. A schematic diagram of the

construct used is shown in Fig. 13. The *celF* gene (SEQ. ID. NO: 26) is modified by PCR using the *celF*pst primer, 5'-ACGCTGCAGTCGCCTGCTCGG-3' (SEQ. ID. NO: 27) and the *celF*xma primer, 5'-CCCCCGGGTCAGGGAGCTCCGGC-3' (SEQ. ID. NO: 28) to generate a recombinant *celF* gene. The recombinant gene has a *Pst*I site at the 5' end and a *Xma*I site at the 3' end (restriction sites are underlined) to facilitate fusion to the VSP leader coding sequence (SEQ. ID. NO: 19) and nopaline synthetase terminator.

The *celF* gene itself contains two internal *Xma*I recognition sites, which are removed by site-directed mutagenesis. Briefly, a portion of the gene containing the sites is subcloned to pBluescript KS+ (Stratagene, La Jolla, California) as a *Bgl* II to *Xho* I fragment. PCR reactions are carried out using primer 2777 (5'-GGCCACCTGGGCAGG-3', SEQ. ID. NO: 29) and the M13-20 sequencing primer (5'-GTAAAACGACGGCCAGT-3', SEQ. ID. NO: 30), thereby destroying the site at 2775 in the Genbank sequence (underline indicates mutated nucleotide).

Similarly, primer 3227 (5'-GCGACGCTCGGGCCG-3', SEQ. ID. NO: 31) and the reverse sequencing primer (5'-AACAGCTATGACCATG-3', SEQ. ID. NO: 32) destroy the site at 3227. The two overlapping amplified fragments are then purified, heated briefly to 95°C and cooled gradually to allow annealing to occur. The annealed template is subjected to another round of PCR using the M13-20 sequencing primer (SEQ. ID. NO: 30) and the reverse sequencing primer (SEQ. ID. NO: 32). This fragment is then subcloned as a *Bgl* II to *Xho* I fragment and sequenced before being used to replace the wild-type *celF* sequence. Both base changes are at the 3rd position in the codon and do not alter protein sequence. The resulting cassette consists of the VSP leader sequence operationally linked to the "mature" portion of the *celF* gene. Subsequent manipulations are carried out as described hereinabove.

The invention is not limited to the preferred embodiments, transformation protocols, transformed plant hosts, and expression constructs explicitly described above, but encompasses all such forms thereof as are encompassed within the scope of the attached claims.

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